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Patricia Andrus

PATENT
Attorney Docket No. 016976-000810US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

CHANG et al.

Application No.: 10/766,993

Filed: January 28, 2004

For: SURFACE EXPRESSION OF BIOLOGICALLY ACTIVE PROTEINS IN BACTERIA

Customer No.: 20350

Confirmation No. 5009

Examiner: Anoop Kumar Singh

Technology Center/Art Unit: 1632

DECLARATION OF QIANG XU, PH.D. UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Qiang Xu, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my Ph.D. in the field of Plant Physiology from Kansas State University in 1991. I am currently a Director, Research at Osel, Inc., the assignee of the present patent application. I have been in this position since 2004.

3. I am a named inventor of the present patent application as well as of US Patent Application No. 10/383,834 ("the '834 application"), now US Patent No. 7,179,458. The claims of the present patent application are directed to an *Lactobacillus jensenii* bacterium comprising an expression cassette for expression of a biologically active protein, wherein the protein is linked to a heterologous carboxyl terminal cell wall targeting region as recited in the claims. I understand that the Examiner has rejected the claims as allegedly anticipated by Tagliabue *et al.* (WO 96/11277). A declaration very similar to this one was filed for the '834 application.

4. To my knowledge, as of the priority date of the '834 application (March 8, 2002), no one had reported actual transformation of *L. jensenii.* Indeed, as discussed in detail in the amendment dated August 22, 2005 for the '834 application, there were several reports in the scientific literature that other *Lactobacillus* species could not be successfully transformed. Therefore, one of ordinary skill in the art would not have assumed that any particular protocol was effective to transform *L. jensenii.* Instead, it is my opinion that it was unpredictable as of the priority date of the '834 application what protocol, if any, would be effective to transform *L. jensenii.* As discussed below, it took considerable effort by the inventors of the '834 application to determine conditions that were effective in generating transformed *L. jensenii.*

5. The inventors of the '834 application made initial attempts to transform *L. jensenii* using several published transformation protocols that had been used successfully for other *Lactobacillus* species. Protocols tested included those described in Bringel *et al.*, *Plasmid* 22:193-202 (1989) and Wei *et al.*, J. *Microbiol. Methods* 21:97-109 (1995). In our experiments, neither of these protocols resulted in successful transformation of *L. jensenii*, further demonstrating that it was not a simple or predictable matter to transform *L. jensenii.*

6. An additional electroporation protocol was identified in Luchansky *et al., J. Dairy Sci.* 74:3293-3302 (1991). Luchansky *et al.* teaches transformation of *L. acidophilus* using a specific electroporation protocol involving plasmid ligation mixtures. See, Luchansky *et al.*, page 3296, paragraph spanning left and right columns. It should be noted at this point that to the extent Tagliabue *et al.* described *any* transformation procedure, it involved transformation with ligation mixtures. *See*, Tagliabue *et al.*, page 11, WO 96/11277). No transformation experiment we have performed using plasmid ligation mixtures or using standard methods (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY) as described in Tagliabue *et al.* have ever resulted in successful transformation of *L. jensenii*. Thus, following the exact protocol and type of DNA (plasmid ligation mixtures) described in Luchansky *et al.* does not result in effective transformation of *L. jensenii.*

7. To achieve transformation of *L. jensenii*, we used intact purified plasmids instead of the ligation mixtures as described in Luchansky *et al.* In addition, instead of using 0.4 cm interelectrode gap cuvettes, per the Luchansky *et al.* protocol, we chose 0.2 cm cuvettes to transform *L. jensenii*. Transformation efficiency of *L. jensenii* was affected by interelectrode gap. *L. jensenii* was cultured to reach 0.7 at OD600 in MRS broth. Cells were washed in sterile distilled H2O and resuspended. Two hundred microliters of competent cells (about $7x10^8$ CFU) were electroporated in 952 mM sucrose and 3.5 mM $MgCl_2$ with 1 μg intact purified plasmid DNA, 2.5 kV, and 200 ohms. After electroporation, bacteria were plated on the erythromycin-containing MRS plates for 24 hours. Then, the erythromycin resistant colonies were counted. As shown in Table I below, this cuvette modification resulted in approximately an eight-fold increase in erythromycin resistant colonies compared to the cuvette size used by Luchansky *et al.*

Table I

Interelectrode gap cuvette (cm)	**Time constant**	**Number of colonies**
0.4	4.7	100

0.2	3.76	790
0.1	5.22	0

9. In view of the forgoing, it is clear that merely following protocols described in the prior art for transforming other *Lactobacillus* species was not effective for transforming *L. jensenii.* It is my scientific opinion that transformation of *L. jensenii* was both unpredictable and difficult prior to the significant experimentation we carried out to generate the data included in the '834 application. Therefore, I do not believe it was obvious for one of ordinary skill in the art how to transform *L. jensenii* as of the priority date of the '834 application.

Date: 6/3/08 By: _____

Qiang Xu, Ph.D.

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PLASMID 22, 193–202 (1989)

Characterization, Cloning, Curing, and Distribution in Lactic Acid Bacteria of pLP1, a Plasmid from *Lactobacillus plantarum* CCM 1904 and Its Use in Shuttle Vector Construction

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A small 2.1-kb plasmid called pLP1 was extracted from *Lactobacillus plantarum* CCM 1904 (ATCC 8014) and cloned into the *Escherichia coli* pUC19 plasmid. As determined by DNA–DNA Southern hybridization with a pLP1-radioactively labeled probe, other lactic acid bacteria such as *L. curvatus, L. sake, Carnobacterium,* and *Leuconostoc mesenteroides* harbor pLP1-related plasmids. Shuttle vectors based on the pLP1 replicon were constructed by inserting the erythromycin-resistance gene from pVA891 into the various pUC19-pLP1 constructions. pLP1-based shuttle vector transformation efficiencies (TE) by electroporation were compared to TE of a broad-host-range plasmid pGK12 in different lactobacilli strains. Expression of the pUC19-pLP1 plasmids in *Escherichia coli* maxicells showed that pLP1 encodes for a 37,000 MW protein which can act in *trans* allowing the replication of plasmids in which this protein is truncated. The pLP1-based shuttle vectors producing this protein replicate in lactobacilli and also in *Bacillus subtilis.* A pLP1-free strain was obtained by incompatibility with a pLP1-based shuttle vector introduced in *L. plantarum* CCM 1904 by electroporation. The absence of pLP1 has no incidence on the strain phenotype suggesting that pLP1 is not essential for the strain in our laboratory conditions.

Lactobacillus plantarum is a Gram-positive bacteria which is commercially important in silage and fermented meat products. The recent success in protoplast transfection (Cosby *et al.,* 1988) and whole cell plasmid transformation by electroporation (Aukrust and Nes, 1988) has made a breakthrough in *L. plantarum* genetics. Increasing our plasmid biology knowledge will provide useful tools for genetic improvement of *L. plantarum* industrial strains. Since the first reports of plasmid occurrence in this genus (Nes, 1984), few studies on *L. plantarum* plasmids have been undertaken: von Husby and Nes (1986) observed plasmid instability in *L. plantarum* starter cultures. Josson *et al.* (1989) revealed plasmids sharing DNA homologies between *L. plantarum* plasmids and pLAB1000, a plasmid isolated from *L. hilgardii,* which is able to replicate in *L. plantarum.*

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pLP1, a small *L. plantarum* plasmid, was characterized and cloned. We assessed the relatedness between the pLP1 plasmid and the 63 lactic acid bacteria DNA, as determined by DNA–DNA hybridization. Since pLP1-related plasmids were found in other lactic acid bacteria, pLP1 could be a good candidate for the construction of shuttle vectors able to replicate in different Gram-positive bacteria. Therefore, pLP1-based shuttle vector replication was tested in *Bacillus subtilis* and *L. plantarum.*

MATERIALS AND METHODS

Strains and Culture Conditions

Lactic acid bacteria strains and their origins are listed in Table 1. Strains from meat or sausage isolated by J. Fournaud were further identified by DNA–DNA hybridization (Champomier *et al.,* 1987; personal communication from M.-C. Montel). Routine characterization of lactobacilli, based on their

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EXHIBIT A

ability to catabolize 49 carbohydrates, was done with an Api 50 CHL gallery as recommended by the manufacturers (Api System S. A.). Lactic acid bacteria were grown at 30°C on MRS medium (de Man *et al.*, 1960). The MRST medium is a MRS broth supplemented with 1% glycine and 0.75 M sorbitol.

Escherichia coli and *B. subtilis* strains were grown at 37°C, with agitation in the LB medium described by Maniatis *et al.* (1982). *E. coli* JM103 (Messing *et al.*, 1981) was used for pLP1 amplification after cloning into the pUC19 plasmid (Norrander *et al.*, 1983). We examined the expression of recombinant plasmids in the maxicell background using the strain *E. coli* CSR603 (Sancar *et al.*, 1979). Replication of the shuttle vectors was studied in the *B. subtilis* strain MI112 (Tanaka and Sakaguchi, 1978). The erythromycin (Em)[2]-resistance gene was taken from the pVA891 plasmid (Macrina *et al.*, 1983). Transformation efficiencies of *L. plantarum* strains obtained with the pULP-type shuttle vectors and with a broad-host-range plasmid, pGK12, of 4.45 kb (Kok *et al.*, 1984), were compared.

Transformation Protocols

E. coli was transformed by the Mandel and Higa (1970) method. Transformants were selected on LB agar plates in the presence of 50 μg/ml ampicillin (Amp) or 100 μg/ml Em. *B. subtilis* was transformed by the Contente and Dubnau (1979) procedure and transformants were selected on LB agar plates containing 5 μg/ml Em. *L. plantarum* was transformed by electroporation following a protocol optimized in our laboratory for the *L. plantarum* CCM 1904 strain. Lactobacilli were grown in MRST broth until an optical density of 0.4 at 600 nm was reached. After washing the cells three times in bidistilled water at room temperature, they were suspended in bidistilled water with 30% polyethylene glycol 1000 (from Sigma) to a final concentration of 1×10^{10} CFU/ml and frozen at −80°C (freezing enhances the transformation efficiency). After thawing, 150 μl of cells mixed with 1 μg DNA was put in a cold 0.2-cm electrode gap Bio-Rad cuvette. A unique pulse was given by the Bio-Rad Gene Pulser with a field strength of 12.5 kV/cm and with the resistance set at 400 Ω using a Bio-Rad Pulse Controller. Cells were then quickly put on ice for 30 min. The suspension was diluted 10 times with MRS and incubated for 4 h at 30°C to allow for the expression of antibiotic resistance and the establishment of plasmid maintenance. Lactobacilli transformants were selected on MRS agar plates supplemented with 2.5 μg/ml Em and 100 μg/ml lincomycin (Lin). Lin was added to the medium to avoid the selection of spontaneous Em-adapted clones that appeared after several days of incubation at a frequency of 10^{-8} (Josson *et al.*, 1989). Transformation efficiency (TE) was defined as the number of Em-resistant transformants per microgram of plasmid DNA per viable cells on MRS agar plates after electroporation.

DNA Extraction, Electrophoresis, and Hybridization Techniques

E. coli plasmid preparation was done by the Triton–lysozyme method (Clewell and Helinski, 1969), and *B. subtilis* plasmid purification by the alkaline lysis method (Niaudet and Ehrlich, 1979). Total lactic acid bacteria DNA was extracted by the Klaenhammer method (1984) with a modification we found to be important: 75 μg/ml of mutanolysine at 37°C instead of lysozyme at 0°C. DNA electrophoresis was conducted on 0.8% agarose gels in Tris–borate buffer, pH 8.2, and restriction enzymes were used as described by Maniatis *et al.* (1982). DNA–DNA hybridization was performed with the Southern (1975) method, and probes were labeled by nick-translation with [α-^{32}P]dCTP (Rigby *et al.*, 1977).

pLP1-curing experiments of *L. plantarum* CCM 1904 were performed according to Caro *et al.* (1984). The presence or absence of the pLP1 plasmid after the curing treatments was

[2] Abbreviations used: Amp, ampicillin; CFU, colony forming unit; Em, erythromycin; Lin, lincomycin; ORF, open reading frame; SDS, sodium dodecyl sulfate; TE, transformation efficiency.

studied by hybridization with a radioactively labeled pLP1 probe on DNA from colonies grown on Hybond C membranes (Amersham). Manufacturers recommendations were followed for DNA fixation on membranes with two important modifications: the clones to be tested were grown for 2 days directly on membranes placed on MRS agar plates in the presence of 2.5% glycine and then were treated with 10% SDS (sodium dodecyl sulfate) before treatment with the denaturing solution.

RESULTS

Cloning of the pLP1 Plasmid and Distribution of Related pLP1 Plasmids among Lactic Acid Bacteria

We studied the extrachromosomal DNA content of 100 strains of lactic acid bacteria from our collection. Total DNA was extracted and analyzed after agarose gel electrophoresis in the presence of ethidium bromide. Most of the strains harbor plasmids. Only 29 strains had no visible plasmid DNA bands: plasmids may exist in these strains but at a very low copy number not detectable under these conditions.

L. plantarum CCM 1904 harbors several plasmids including a 2.1-kb plasmid, pLP1, we chose to study. pLP1 is one of the smallest plasmids we observed among all the lactic acid bacteria plasmids and therefore was easier to analyze. In order to clone the pLP1 plasmid, DNA of a 1-liter *L. plantarum* CCM 1904 batch culture was extracted and submitted to a 0.8% agarose gel electrophoresis in the presence of ethidium bromide. The band corresponding to the covalently closed circular form of the pLP1 plasmid was cut out and DNA was separated from the agarose gel by electroelution (Maniatis *et al.*, 1982). Several restriction enzymes present in the polylinker of the cloning plasmid pUC19 were tested on the pLP1 plasmid. Two restriction enzymes, *Hin*cII and *Eco*RI, were selected for cloning because they linearize pLP1 in two unique sites separated by 1 kb (Fig. 1). The restriction enzyme *Hin*dIII was used to verify the pLP1 orientation in the cloning vector pUC19. Four recombinant plasmids were constructed: pULP1, pULP2, pULP21, and pULP22 (Fig. 1).

Southern hybridization between total DNA of 63 strains with labeled pLP1 detected 9 other strains harboring homologous sequences with pLP1 (Fig. 2 and Table 1). Hybridization with pLP1 occurred with three other *L. plantarum*, but not with all of the *L. plantarum* tested. Six strains isolated from meat products harbor plasmids which share DNA homology with pLP1: one *L. curvatus*, two *L. sake*, two *Carnobacterium*, and one *Leuconostoc mesenteroides*.

L. plantarum CCM 1904 pLP1-Curing Attempts by Chemical and Physical Agents

Several curing agents with specific mechanisms, successfully used in the curing of other Gram-positive bacteria plasmids (Caro *et al.*, 1984), were tested on *L. plantarum* CCM 1904. A physical agent (temperature) and chemical agents such as intercalating dyes (acriflavine, acridine orange, and ethidium bromide), antibiotics (rifampicin and novobiocin), an ionic surface-active agent (SDS), and a mutagen (mitomycin C) were ineffective at sublethal concentrations (data not shown). Chassy *et al.* (1978) employed curing agent combinations to cure the lactose plasmid in a *L. casei* strain. In our case, combinations at various sublethal concentrations (indicated in μg/ml) of acriflavine (1, 2.5, 5) and mitomycin C (1, 2), acridine orange (7.5, 10) and rifampicin (0.5, 1), and ethidium bromide (5, 7.5) and novobiocin (0.25, 0.5) were also ineffective.

Construction of the Shuttle Vectors

The Amp minimal inhibitory concentration (1 μg of Amp/ml) of *L. plantarum* remained unchanged even when the pULP-type plasmid (carrying the pUC19 Amp-resistance gene) was present in the bacteria. Therefore, the pUC19 Amp-resistance gene is not expressed in *L. plantarum*. In order to test for the replication of pLP1 in Gram-positive bacteria, the Em-

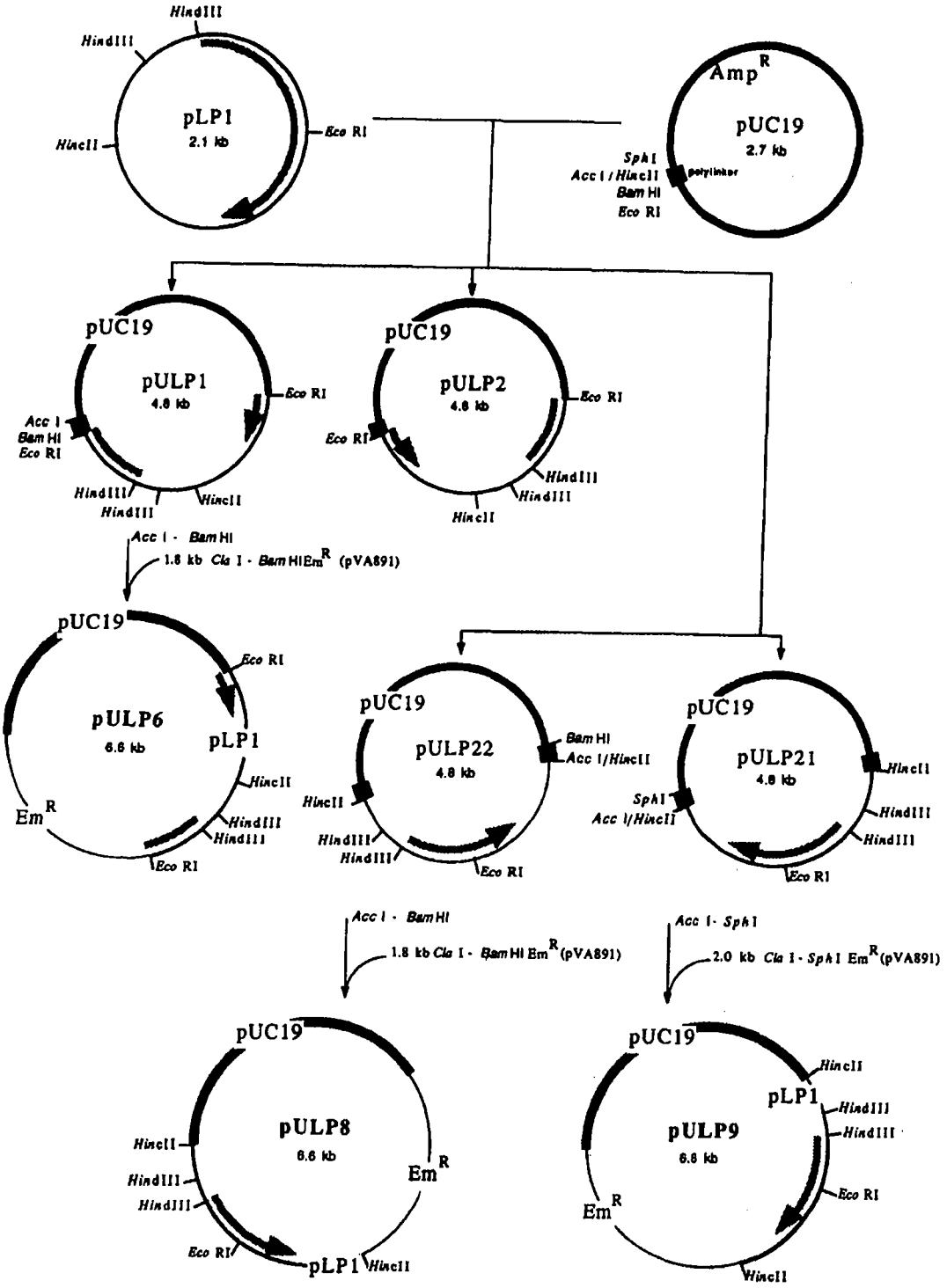

FIG. 1. Cloning of the pLP1 plasmid and construction of the shuttle vectors. Amp^R, ampicillin-resistance gene of the pUC19 plasmid; Em^R, erythromycin-resistance gene taken from the plasmid pVA891. Grey arrows, position and direction of the major open reading frame in pLP1.

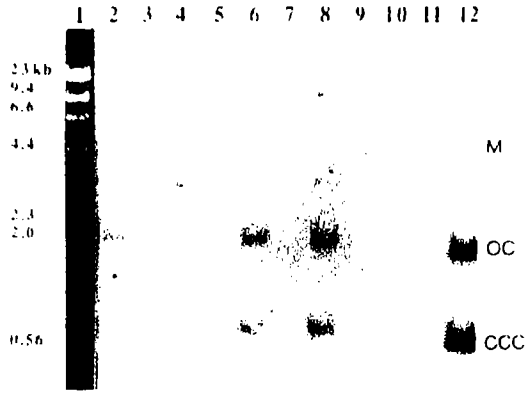

FIG. 2. Presence of pLP1-related plasmids in various lactic acid bacteria strains. Lane 1, λ phage DNA digested by *Hin*dIII restriction endonuclease after DNA agarose gel electrophoresis in the presence of ethidium bromide. DNA/DNA Southern hybridization with the pLP1-labeled plasmid; *Lactobacillus* plasmids were not digested with restriction endonucleases. Lane 2, *L. sake* 160 × 1; 3, *L. sake* 52; 4, *L. curvatus* S9; 5, *L. sake* 18; 6, *L. sake* 207; 7, *Leuconostoc mesenteroides* BGI; 8, *Leuconostoc mesenteroides* 318; 9, *L. curvatus* 324; 10, *L. curvatus* 204; 11, *Carnobacterium* 209; and 12, *L. plantarum* CCM 1904 from which pLP1 has been isolated. The labeled bands in lane 4 differ distinctly from the pLP1 bands (lane 12). The letters refer to the plasmid DNA form; M, multimer; CCC, covalently closed circular, and OC, open circular.

resistance gene from pVA891, expressed in both Gram-negative and Gram-positive bacteria, was inserted in pULP1, pULP21, and pULP22 which gave the shuttle vectors pULP6, pULP9, and pULP8, respectively (see Fig. 1 for cloning details). pULP2 was not studied further because of structural modifications occurring in the *E. coli* JM103 strain.

Expression of pULP-Type Plasmids in a Gram-Negative Bacteria Background

E. coli maxicell strain CSR603 was transformed by the pUC19, pULP1, pULP21, and pULP22 plasmids. The transformants were selected for Amp resistance. One specific pLP1 protein of 37,000 MW was only synthesized with pULP21 or pULP22 plasmids (Fig. 3). Analysis of the sequence of pLP1 (accompanying paper by A. Bouia *et al.*) shows one open reading frame (ORF) that could encode such a protein. This protein was expressed in the *E. coli* background by its own promoter region (the two ORF orientations lead to the same result). The ORF contains the *Eco*RI unique restriction site into which pUC19 was inserted for the pULP1 plasmid construction. In the case of pULP1, a protein around 20,000 MW present in a diffuse band was obtained. From the sequence data, we deduced that this protein contains the beginning of the 37,000 MW protein and 20 amino acids of the β-galactosidase (from pUC19).

Replication of the pULP-Type Shuttle Vectors in a Heterologous Gram-Positive Environment

A plasmid-free *B. subtilis* strain MI112 was transformed with pULP6, pULP8, and pULP9 plasmids. Transformants were selected for Em resistance. No transformants were obtained with pULP6, a plasmid whose ORF has been interrupted with an insertion (see Fig. 1). On the other hand, pULP8 or pULP9 replicates in *B. subtilis*. The complete 37,000 MW protein expressed in the *E. coli* maxicell strain CSR603 seems necessary for the pULP-type plasmid replication in *B. subtilis*.

Replication of the pULP-Type Shuttle Vectors in L. plantarum and pLP1 Curing from L. plantarum CCM 1904

L. plantarum CCM 1904 is transformed by the plasmids pULP8 and pULP9. A plasmid analysis on agarose gel of the transformants (Fig. 4) revealed that pULP8 or pULP9 entry leads to the loss of the endogenous pLP1 plasmid. Southern hybridization with a labeled pULP8 probe verified the substitution of pLP1 by pULP8. Plasmids extracted from those transformants conferred Amp and Em resistance to *E. coli* and their endonuclease restriction patterns were those of pULP8 or pULP9.

Segregational instability was examined: 95% of the pULP8 and pULP9 *L. plantarum* CCM 1904 transformants lose their Em-resistance phenotype after 20 generations in a nonselective liquid medium (MRS without Em). *L. plantarum* FB1000 is one of the Em-sensitive clones which is pLP1- and pULP8-free (Fig. 4, lane 3). No differences in morphology,

TABLE I

PRESENCE OF RELATED pLP1 PLASMID IN LACTIC ACID STRAINS FROM CULTURE COLLECTIONS OR ISOLATED FROM FERMENTED PRODUCTS

Lactic acid bacteria strains		Origin[a]	Hybridization with labeled pLP1[b]
Carnobacterium	15	1	–
Carnobacterium	182	1	–
Carnobacterium	185	1	–
Carnobacterium	208	1	+
Carnobacterium	209	1	–
Carnobacterium	210	1	–
Carnobacterium	211	1	–
Carnobacterium	213	1	–
Carnobacterium	320	1	–
Carnobacterium	328	1	+
Carnobacterium	332	1	–
Carnobacterium divergens	327	1	–
Carnobacterium divergens	329	1	–
Lactobacillus bavaricus	330	1	–
Lactobacillus casei alactosus		3	–
Lactobacillus casei	64 H	3	–
Lactobacillus cellobiosus	ATCC 11739		–
Lactobacillus curvatus	204	1	–
Lactobacillus curvatus	324	1	–
Lactobacillus curvatus	318 B	1	–
Lactobacillus curvatus	S9	1	+
Lactobacillus delbrueckii var. bulgaricus	NCDO 1489	3	–
Lactobacillus delbrueckii var. leichmannii	NCDO 299	3	–
Lactobacillus gasserii	NCDO 3	3	–
Lactobacillus jugurti	NCDO 100	3	–
Lactobacillus pentosus	NCFB 363		–
Lactobacillus plantarum	H (commercial strain)	4	–
Lactobacillus plantarum	L (commercial strain)	4	–
Lactobacillus plantarum	A	5	+
Lactobacillus plantarum	61 D	3	+
Lactobacillus plantarum	61T	3	+
Lactobacillus plantarum	LP 85-2 (CIP I 820)	6	–
Lactobacillus plantarum	ATCC 14917		–
Lactobacillus plantarum	CCM 1904 (ATCC 8014)		+
Lactobacillus plantarum	FB 1000	This work	–
Lactobacillus plantarum	NCIB 8299		–
Lactobacillus sake	18	1	–
Lactobacillus sake	35	1	–
Lactobacillus sake	52	1	–
Lactobacillus sake	53	1	–
Lactobacillus sake	63	1	–
Lactobacillus sake	67	1	–
Lactobacillus sake	110	4c	–
Lactobacillus sake	173	1	–
Lactobacillus sake	174	1	–
Lactobacillus sake	175	1	–
Lactobacillus sake	179	1	–
Lactobacillus sake	180	1	–
Lactobacillus sake	184	1	–
Lactobacillus sake	186	1	–
Lactobacillus sake	207	1	+

TABLE 1—*Continued*

Lactic acid bacteria strains		Origin[a]	Hybridization with labeled pLP1[b]
Lactobacillus sake	231	4d	–
Lactobacillus sake	319	1	–
Lactobacillus sake	160 × 1	2	+
Lactobacillus sake	160 × 13	2	–
Lactobacillus sake	G13	1	–
Lactobacillus salivarius var. salivarius	ATCC 11741	7	–
Leuconostoc mesenteroides	G1	1	–
Leuconostoc mesenteroides	G4	1	–
Leuconostoc mesenteroides	G14	1	–
Leuconostoc mesenteroides	BG1	1	–
Leuconostoc mesenteroides	318	1	+
Leuconostoc cremoris		7	–
Leuconostoc lactis		7	–

[a] Origin of 1, INRA Jouy en Josas Dr. Fournaud's collection (isolated from sausage meat); 2, INRA Jouy en Josas Dr. Fournaud's collection (isolated from horse meat); 3, F. Gasser Institut Pasteur, 28 rue de Dr Roux 75724 Paris, France; 4, INRA Theix collection (c, isolated from sausage meat; d, isolated from pork); 5, isolated in our laboratory from bread dough, 6, isolated from silage at the laboratory of Microbiologie et Génétique Appliquées, 118; route de Narbonne 31062 Toulouse, France; 7, from the API collection, La Balme-les-Grottes, 38390 Montalieu-Vercieu, France.

[b] –, No hybridization was detected; +, hybridization was detected.

growing abilities, or fermentation patterns of 49 carbohydrates (Api gallery) could be detected between *L. plantarum* CCM 1904 and *L. plantarum* FB1000. The 2.1-kb pLP1 plasmid is not essential for bacterial growth under our cultural conditions. *L. plantarum* is transformed by pULP8 and pULP9 with the same efficiency independently of pLP1 presence (Table 2). Plasmid pULP6 does not transform *L. plantarum* FB1000. A functional protein encoded by the ORF (expressed in *E. coli* as a 37,000 MW protein) seems to be necessary for pULP-type shuttle vector replication in *L. plantarum*. The pULP6 plasmid is then able to replicate in *L. plantarum* CCM 1904 because the 37,000 MW protein, potentially encoded by the endogenous pLP1 plasmid, acts in *trans* on the pULP6 plasmid replication. The pLP1 plasmid is retained in all of the 10 transformants tested harboring pULP6 (data not shown), but not in those harboring pULP8 and pULP9.

Comparisons of the pULP-Type Shuttle Vector Transformation Efficiency

Luchansky *et al.* (1988) studied the TE of different plasmids in *L. acidophilus* NCK89 and showed that pGK12 was the most efficient (10^3 to 10^4 transformants/μg of DNA). In order to compare TE, lactobacilli strains were

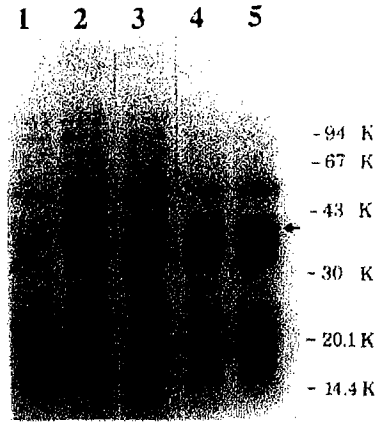

FIG. 3. Autoradiography of a polyacrylamide SDS gel of [^{35}S]methionine-labeled plasmid proteins in uv-irradiated *E. coli* CSR603 maxicells. Protein molecular weights have been estimated using the LMW calibration kit (Pharmacia, France). Lane 1, *E. coli* CSR603; CSR603 transformed with 2, pUC19; 3, pULP1; 4, pULP21; and 5, pULP22. A 37,000 MW protein is seen only when the pLP1 plasmid has been inserted in the pUC19 *Hin*cII restriction site (pULP21 and pULP22). A 20,000 MW protein band is specifically encoded by the pULP1 plasmid when pLP1 has been inserted in the pUC19 *Eco*RI restriction site.

transformed by the pGK12 plasmid and by the pULP-type shuttle vectors (Table 2). The pULP8 or pULP9 plasmids transform *L. plantarum* NCIB 8299 with the same efficiency (5000 transformants/μg of DNA). Tenfold more transformants are obtained with pGK12 than with pULP8. Plasmid pULP6 transforms *L. plantarum* CCM 1904 with a TE of 1×10^{-6} which is about 100 times less than with pGK12.

TABLE 2

TRANSFORMATION EFFICIENCY[a]

Tested strains	pULP8	pULP9	pGK12
L. plantarum CCM 1904	2×10^{-7}	2×10^{-7}	7×10^{-5}
L. plantarum FB1000	3×10^{-7}	3×10^{-7}	4×10^{-5}
L. plantarum NCIB 8299	1×10^{-5}	1×10^{-5}	1×10^{-4}

[a] Number of transformants per microgram of plasmid and per viable cell after electroporation.

DISCUSSION

pLP1 and pLP1-related plasmids are found in several lactic acid bacteria species including lactobacilli isolated from meat products. Since plasmid pLP1 may represent an important plasmid type in lactic acid bacteria, pLP1 genetic investigations become of major interest. Therefore, pLP1 seems to be a good candidate for shuttle vector constructions which will allow gene transfer in lactic acid bacteria.

pLP1-based shuttle vectors are able to replicate in *L. plantarum* and *B. subtilis*. However, inserting 4.5-kb DNA in the pLP1 plasmid decreased considerably its segregational stability even if the pLP1 plasmid is very stable in *L. plantarum* CCM 1904 (all the attempts to cure pLP1 with different chemicals failed). The pULP-type vectors are lost in *L. plantarum* after 20 generations without selective pressure, strongly limiting their use as a gene

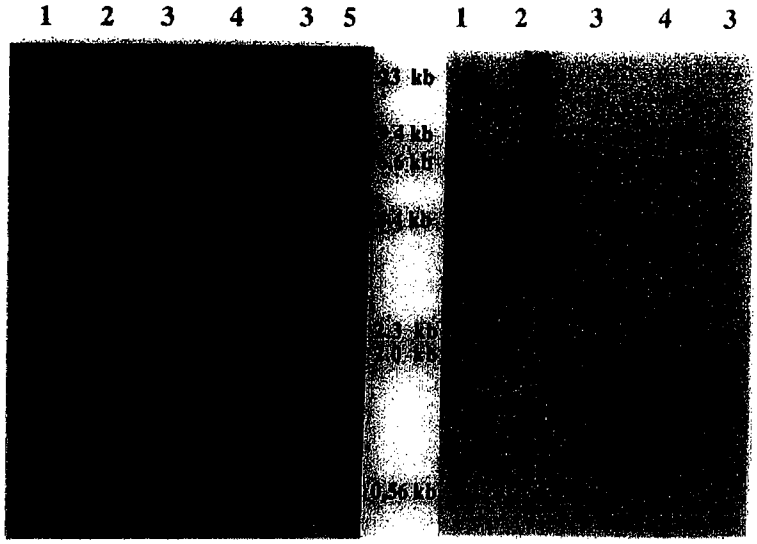

FIG. 4. Agarose gel electrophoresis in the presence of ethidium bromide of not-digested plasmid DNA from *L. plantarum* (left); Southern hybridization with the radioactively labeled pULP8 plasmid (right). Lane 1, Em-resistant *L. plantarum* CCM 1904 after transformation with the pULP8 plasmid; 2, pULP8 extracted from *E. coli* used as transforming DNA; 3, *L. plantarum* FB1000; 4, *L. plantarum* CCM 1904 (the covalently closed circular form of the 2.1-kb pLP1 plasmid can been clearly seen); 5, λ phage DNA digested by *Hin*dIII restriction endonuclease. Entry of pULP8 in *L. plantarum* CCM 1904 provoked the loss of the endogenous pLP1 plasmid. In a nonselective liquid medium, the plasmid pULP8 is also lost and a pLP1- and pULP8-free strain is obtained: *L. plantarum* FB1000 (this strain is not plasmid free as can be seen on lane 3). HMW (high-molecular-weight forms) of pULP8 plasmid can be seen.

transfer tool in industrial strains which are grown on complex media.

Some plasmids of Gram-positive bacteria replicate via single-stranded plasmid DNA intermediates (te Riele *et al.*, 1986). Data discussed by Bouia *et al.* (accompanying paper) suggest that pLP1 is also a ssDNA plasmid replicating via a rolling-circle replicative mechanism. Gruss and Ehrlich (1988) demonstrated that insertion of foreign DNA (such as pUC-type plasmid) in ssDNA plasmids resulted in the generation of high-molecular-weight plasmid multimers: such forms can also be seen in our case (Fig. 4). The segregational instability obtained with the plasmids pULP8 and pULP9 could be explained by pLP1 replicative mechanism. Effects of DNA inserts on the segregational instability of a ssDNA plasmid, pUB110, has been demonstrated by Bron *et al.* (1988).

Comparisons between the lactobacilli TE obtained with the pULP-type shuttle vectors and those with the heterologous replicon pGK12 from *Lactococcus cremoris* showed that the homologous pULP-type vectors transform *L. plantarum* NCIB 8299 10 times more efficiently than the pGK12 plasmid. Incompatibility and copy number of the resident plasmid may affect the efficiency of DNA transformation (van der Lelie *et al.*, 1988). However, incompatibility between the endogenous pLP1 plasmid and the pLP1-based shuttle vectors did not affect TE since *L. plantarum* CCM 1904 and FB1000 (the pLP1-free *L. plantarum* CCM 1904 strain) are transformed by pULP8 or pULP9 with the same efficiency. Optimization of the TE by electroporation of *L. plantarum* CCM 1904 is currently in progress.

An interesting feature of this work is the demonstration that the integrity of the protein encoded by the ORF is required for pLP1-based plasmid replication in lactobacilli and in *B. subtilis*. Plasmid pULP6 (the ORF of pLP1 is interrupted by an insertion so that the functional protein cannot be expressed) replicates only in the presence of pLP1 which produces the replication protein acting in *trans* on the pULP6 replication. Thus, pULP6 does not transform *L. plantarum* FB1000. This strain was obtained after transformation by pULP8. When plasmids are not lost after chemical or physical treatments, incompatibility between endogenous plasmids and shuttle vectors can be a good curing alternative.

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An improved method for the transformation of *Lactobacillus* strains using electroporation

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Abstract

Because of their widespread industrial and medical importance, there is considerable interest in the manipulation and improvement of *Lactobacillus* strains using modern genetic engineering techniques. However, most reports have focused on industrial strains and often have resulted in non-reproducible transformation efficiencies. We have developed an optimised protocol for electroporating foreign plasmid DNA into clinical strains of lactobacilli. Treatment of the recipient lactobacilli with either lysozyme, glycine or penicillin improved electrotransformation efficiencies up to 480-fold. A critical step in achieving efficient and reproducible electrotransformation of clinical lactobacilli with the plasmid pSA3 was the requirement for a post-pulse recovery time of 2–3 h, combined with the use of sub-inhibitory concentrations of antibiotics in the selective plates. While pNZ17 transformants also benefited from a post-pulse recovery period, good transformation efficiencies could be achieved when plated directly onto selective concentrations of chloramphenicol. We also observed significant differences in electrotransformation efficiencies between our guinea pig vaginal *Lactobacillus* isolates (maximum of 4.8×10^4 transformants/μg pNZ17 DNA) and the human *L. casei* strain ATCC 393 (3.7×10^6 transformants/μg pNZ17 DNA). An optimised procedure for the electroporation of plasmid DNA into lactobacilli is described.

Keywords: Electroporation; Cell-wall modification; *Lactobacillus*; Plasmid; Transformation

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EXHIBIT B

1. Introduction

Bacteria of the genus *Lactobacillus* have considerable industrial and medical importance. They are well known for their widespread application in various food and agricultural fermentation processes [1]. Medically, lactobacilli are found as part of the normal microbial flora in healthy humans where they inhabit and proliferate as commensals at mucosal surfaces such as the gastrointestinal and urogenital tracts [2–5]. Lactobacilli at these sites have been recognised as playing an important role in the regulation of the body's normal microflora [6] and as a consequence, several species of lactobacilli have been used as probiotics to benefit the health of humans and animals [2,7–9]. This fact, combined with the natural adjuvant effect of the *Lactobacillus* cell wall, has led several groups [10,11] to consider the use of genetically-modified strains of lactobacilli to deliver foreign proteins (vaccine antigens, enzymes, hormones) to both the gastrointestinal and urogenital tracts of humans.

In view of the widespread industrial and medical applications of lactobacilli there has been considerable interest in strain improvement and in genetic transfer systems for *Lactobacillus* species. Essential prerequisites for the genetic engineering of these strains are that the methods must be convenient and reliable. Until recently, the only routes available for genetic transformation of lactobacilli were via conjugation or protoplast transformation. These not only resulted in low transformation efficiencies, but were also tedious and seldom reproducible [12–14]. A breakthrough in the transformation of *Lactobacillus* strains was made when Chassy and Flickinger used electroporation to introduce plasmid and phage DNA into *L. casei* [15]. Since then, methods for the introduction of plasmid DNA into several *Lactobacillus* species have been developed [12–14,16–23] although few studies have examined the optimisation of this procedure for lactobacilli [24,25]. The *Lactobacillus* strains which have received most attention to date are those of industrial significance [1,26] with no reports optimising conditions for the electrotransformation of plasmid DNA into medical isolates of lactobacilli.

In this study we demonstrate the use of electroporation as an efficient and reproducible method for the genetic transformation of different lactobacilli species of medical importance. Four strains of lactobacilli (three isolated from the vaginal tract of guinea pigs and one human isolate) and two broad host-range shuttle plasmid vectors (pNZ17 and pSA3) were used. Parameters investigated included (i) pre-treatment of recipient cells with lysozyme, glycine and penicillin, (ii) extended post-pulse incubation times and (iii) the use of sub-inhibitory concentrations of antibiotics in the selective media. Plasmid origin also was investigated for its effect on transformation efficiencies in these lactobacilli.

2. Materials and methods

2.1. Bacterial strains and plasmids

Four *Lactobacillus* strains were used. *Lactobacillus* sp. BR3, *L. acidophilus*

BR9 and *L. fermentum* BR11 were isolated in our laboratory from the guinea pig vaginal tract. *L. casei* ATCC 393 is a human isolate. Two broad host-range plasmid vectors were used. pNZ17 is a low copy number vector which contains the origin of replication derived from the cryptic *Streptococcus lactis* plasmid pSH71 [27]. It is 5.7 kb in size and contains genes for resistance to chloramphenicol and kanamycin. pSA3 also is a broad host-range vector, derived from the *Escherichia coli* plasmid pACYC184 and the *Streptococcus sanguis* plasmid pGB305 [28]. It is 10.2 kb in size and contains resistance genes for chloramphenicol, erythromycin and tetracycline. *E. coli* JM109 was used as the initial host strain for plasmids pNZ17 and pSA3. Plasmid DNA was prepared by using maxipreparation or minipreparation methods [29] and purified by using polethylene glycol precipitation [29].

2.2. Preparation of recipient bacteria for electroporation

Untreated lactobacilli were used in initial experiments in this study and were prepared as follows. A stationary phase (16–18 h) culture of the recipient *Lactobacillus* strain was inoculated (2% inoculum) into 10 ml of MRS broth (Oxoid) and incubated in 5% CO_2 at 37°C without shaking. The cells were harvested in the early-log phase (OD_{660} 0.2–0.3, usually after 3 h incubation), chilled on ice for 5 min and washed twice with ice-cold washing buffer (5 mM sodium phosphate, pH 7.4, 1 mM $MgCl_2$).

2.2.1. Penicillin treatment

A stationary phase (16–18 h) culture of recipient *Lactobacillus* was inoculated into 10 ml of MRS broth (2% inoculum) and incubated in 5% CO_2 for 2 h at 37°C without shaking (OD_{660} 0.1–0.2). Penicillin then was added to a final concentration of 0.1, 0.5, 1, 5, 10, or 20 μg/ml and incubation was continued for a further 1–2 h (OD_{660} 0.2–0.3). Cells were harvested and washed, as above.

2.2.2. Lysozyme treatment

Recipient strains were grown in MRS broth and harvested as described above. The cells were resuspended in 1 ml washing buffer and warmed to 37°C. Egg white lysozyme (Boehringer Mannheim) was added to a final concentration of 10 μg/ml and incubation was continued at 37°C for 30 min. Cells were collected by centrifugation (6,000 × g, 5 min at 4°C) and washed once in ice-cold washing buffer.

2.2.3. Glycine treatment

An early stationary phase (16–18 h) culture of recipient *Lactobacillus* was inoculated into 10 ml of MRS broth (2% inoculum) supplemented with 1% glycine (w/v). The culture was grown for 3 h (OD_{660} 0.2–0.3), harvested and washed, as described above.

2.3. Electroporation

A Gene Pulser™ apparatus (Bio-Rad Laboratories, Richmond, CA) was used. Briefly, 1 μl of plasmid DNA (0.5 μg of either pNZ17 or pSA3) was mixed with 50 μl of the ice-cold cell suspension in a Gene Pulser™ disposable cuvette (inter-electrode distance 0.2 cm) and held on ice for at least 2 min. This mixture then was exposed to a high-voltage electric pulse (peak voltage of 2.5 kV, capacitance of 25 μF, parallel resistance of 400 Ω) which delivered a peak field strength of 12.5 kV/cm and produced a pulse duration of 7.4–9.4 ms for untreated recipient bacteria.

2.4. Recovery and plating of electrotransformants

Following electroporation the bacterial cells either were (i) allowed to recover in non-selective media for various lengths of time and then plated onto selective media, or (ii) plated directly onto MRS agar containing chloramphenicol or erythromycin. Routine selection for pNZ17-containing transformants involved plating out the electroporation mix directly onto MRS agar containing 5 μg/ml of chloramphenicol. However, modifications were made for selecting pSA3-containing transformants where the cells first were plated onto sub-inhibitory concentrations (0.5 or 3.0 μg/ml) of antibiotics followed by replica plating onto agar at the selective chloramphenicol concentrations of 3.0 or 5.0 μg/ml, depending on the recipient strain.

2.5. Comparison of field strength and suspending buffer

All cells (pre-treated or untreated) were resuspended in either: (i) S buffer (0.5 M sucrose); (ii) SM buffer (0.3 M sucrose, 1 mM $MgCl_2$, pH 7.4); (iii) SSM buffer (0.3 M sucrose, 5 mM sodium phosphate, pH 7.4, 1 mM $MgCl_2$); or (iv) 3 × SM buffer, at a cell concentration of 10^{8-9} colony forming units (CFU)/ml. These cells were held on ice and used within 30 min. We also compared the transformation efficiency and survival rate of *L. fermentum* BR11 cells electroporated with pNZ17 at several field strengths (5.0, 7.5, 10.0 and 12.5 kV/cm). Transformation efficiency was expressed as the number of CFU per μg of plasmid DNA.

2.6. Confirmation of electrotransformants

For the confirmation of electrotransformants, plasmid DNA was isolated from lactobacilli using a modification of the cell lysis method of Chassy and Giuffrida [30] followed by chromosomal DNA denaturation, protein extraction and plasmid DNA precipitation with isopropanol. These plasmid preparations were analysed firstly by electrophoresis in 0.7% agarose gels and stained with ethidium bromide, and secondly by Southern blotting onto nylon membrane and hybridisation with ^{32}P-labelled plasmid probe [29].

3. Results

3.1. Comparison of electric field strength and buffer system on the electrotransformation of Lactobacillus fermentum BR11 with pNZ17

L. fermentum BR11 with intact cell walls was used in preliminary experiments to evaluate the effect of electric field strength on both survival rate and transformation efficiency (Table 1). The highest numbers of transformants (up to 180 CFU/μg DNA) were obtained with a field strength of 12.5 kV/cm, with 32% cell survival being achieved under these conditions. Lowering the field strength reduced the number of transformants, although there was less total cell killing at the lower levels tested (75% cell survival at 5.0 kV/cm). Electrotransformants were obtained using each of the four buffer systems tested (S, SM, 3 × SM, SSM). Whilst the differences observed in the various buffer systems were not large (ranging from 75 CFU/μg DNA for SSM buffer to 180 CFU/μg DNA for 3 × SM buffer), the best and most reproducible results were obtained with 3 × SM buffer (data not shown). Consequently, standard conditions adopted for all subsequent transformations included the use of 3 × SM buffer, the buffer system that was used in this experiment, and a peak field strength of 12.5 kV/cm.

3.2. Effect of cell wall modification on the electrotransformation of L. fermentum BR11 with pNZ17 and pSA3

Electrotransformation efficiencies obtained using standard conditions (3 × SM buffer, 12.5 kV/cm) but with untreated lactobacilli were low and often not

Table 1
Effect of field strength on the electrotransformation of *Lactobacillus fermentum* BR11 with pNZ17

Field strength (kV/cm)	Survival rate (%)	Transformation efficiency (CFU/μg plasmid DNA)	Mean ± SD (n = 3)
5.0	75	0	
		0	6.7 ± 6.7
		20	
7.5	50	0	
		0	6.7 ± 6.7
		20	
10.0	42	12.0	
		15	22.3 ± 8.9
		40	
12.5	32	0	
		120	100 ± 53
		180	

Untreated cells of *L. fermentum* were grown in MRS to an OD_{660} of 0.2–0.3, washed and exposed to field strengths of 5.0–12.5 kV/cm in a Bio-Rad Gene Pulser™ (interelectrode distance 0.2 cm). pNZ17-transformants were selected on chloramphenicol plates. Transformation efficiencies are presented for three separate experiments.

reproducible (ranging from 0 to 180 transformants per μg DNA, Table 1). For Gram-positive bacteria such as lactobacilli, the structure and composition of the cell wall is considered to be a major hindrance to successful transformation [31–33]. We therefore evaluated the effect of various treatments designed to weaken the cell wall of *L. fermentum* BR11, on electrotransformation efficiency with both plasmids pNZ17 and pSA3.

3.2.1. Penicillin

Exponentially-growing cells of *L. fermentum* BR11 were treated with different concentrations of penicillin for 1–2 h and then electrotransformed using standard conditions. Transformants were selected on plates containing 0.5 μg/ml of either Cm or Em and then replica plated onto selective plates with 3 μg/ml of Cm and 5 μg/ml of Em. Fig. 1 shows that penicillin treatment significantly improved the transformation efficiencies for both pNZ17 and pSA3. The optimal concentration of penicillin for both plasmids was found to be 10 μg/ml. This resulted in a 480-fold improvement of transformation efficiency for pNZ17 (maximum of 4.8×10^4 transformants/μg DNA) compared to non-penicillin treated controls. Increasing the penicillin concentration further to 20 μg/ml subsequently reduced the electrotransformation efficiency ($1.8–2.3 \times 10^2$ CFU/μg DNA). Whereas electrotransformation of untreated cells was highly variable (sometimes complete-

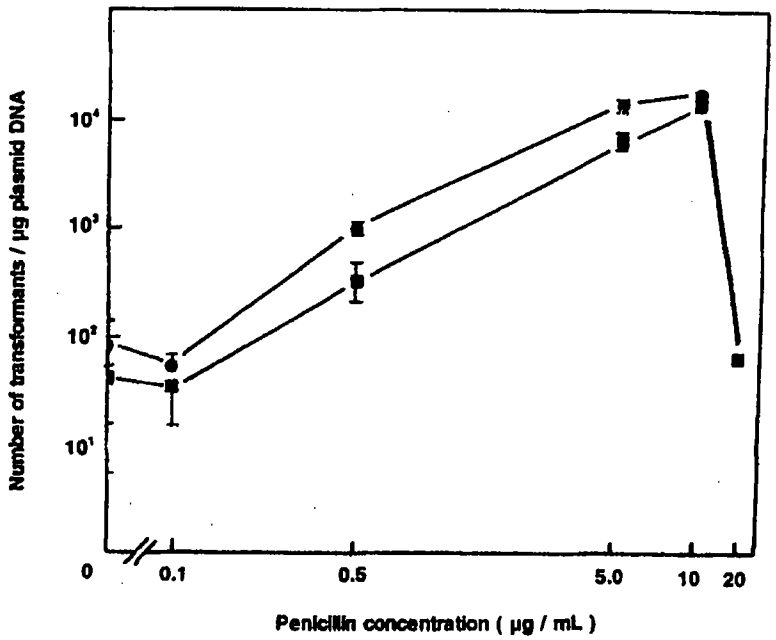

Fig. 1. Effect of penicillin treatment on the electrotransformation of *Lactobacillus fermentum* BR11 with plasmids pNZ17 (●) and pSA3 (■). Recipient cells were treated with 0.1, 0.5, 1.0, 5.0, 10 and 20 μg/ml of penicillin, resuspended in 3 × SM buffer and electroporated at a peak field strength of 12.5 kV/cm. Transformants were selected on 0.5 μg/ml of either Cm or Em and then replica plated onto selective plates with 3 μg/ml of Cm and 5 μg/ml of Em.

ly unsuccessful), penicillin-treated cells always could be electrotransformed successfully with both plasmids.

3.2.2. Lysozyme

When *L. fermentum* BR11 was treated with lysozyme (10 μg/ml) for 30 min at 37°C, a 15-fold increase in electrotransformation was observed (Table 2). Three other *Lactobacillus* strains tested also produced significantly higher electrotransformation efficiencies when treated with lysozyme (data not shown). As with penicillin treatment, lysozyme treatment produced reproducible transformation compared with untreated cells. The best transformation efficiency achieved with lysozyme treatment of *L. fermentum* BR11 was 2.4×10^3 transformants per μg pNZ17 DNA (Table 2).

3.2.3. Glycine

When recipient cells were grown in MRS broth containing 1% glycine, transformation efficiencies consistently were improved by at least 170-fold compared to untreated cells (Table 2). Whilst glycine treatment was not quite as successful as penicillin treatment, it did result in transformation efficiencies of $2.5–2.7 \times 10^4$ *L. fermentum* BR11 transformants per μg of pNZ17 DNA.

3.3. Effect of the recipient bacterial strain on electrotransformation efficiency

The recipient bacterial strain had a major effect on the electrotransformation efficiency with the plasmid pNZ17, even when the bacterial cell walls were weakened by treatment with penicillin. All three guinea pig vaginal lactobacilli tested (BR3, BR9, BR11) had similar transformation efficiencies of between 1.5 and 4.8×10^4 CFU/μg DNA. However, the human *L. casei* isolate tested had a 100-fold higher transformation efficiency with this plasmid (3.7×10^6 CFU/μg DNA). The authenticity of both pNZ17 (Fig. 2) and pSA3 (data not shown) transformants was confirmed both by gel electrophoresis of plasmid DNA isolations and by DNA hybridisation studies.

Table 2
Effect of cell wall modification on the electrotransformation of *Lactobacillus fermentum* BR11 with pNZ17

Cell wall treatment	Transformants per μg pNZ17 DNA
Untreated	$0–1.6 \times 10^2$
Penicillin	$4.5–4.8 \times 10^4$
Lysozyme	$2.1–2.4 \times 10^3$
Glycine	$2.5–2.7 \times 10^4$

Untreated lactobacilli were compared with cells grown for 1–2 h in the presence of 10 μg/ml penicillin, cells treated with 10 μg/ml lysozyme for 30 min and cells grown for 3 h in the presence of 1% glycine. pNZ17 transformants were selected on chloramphenicol plates.

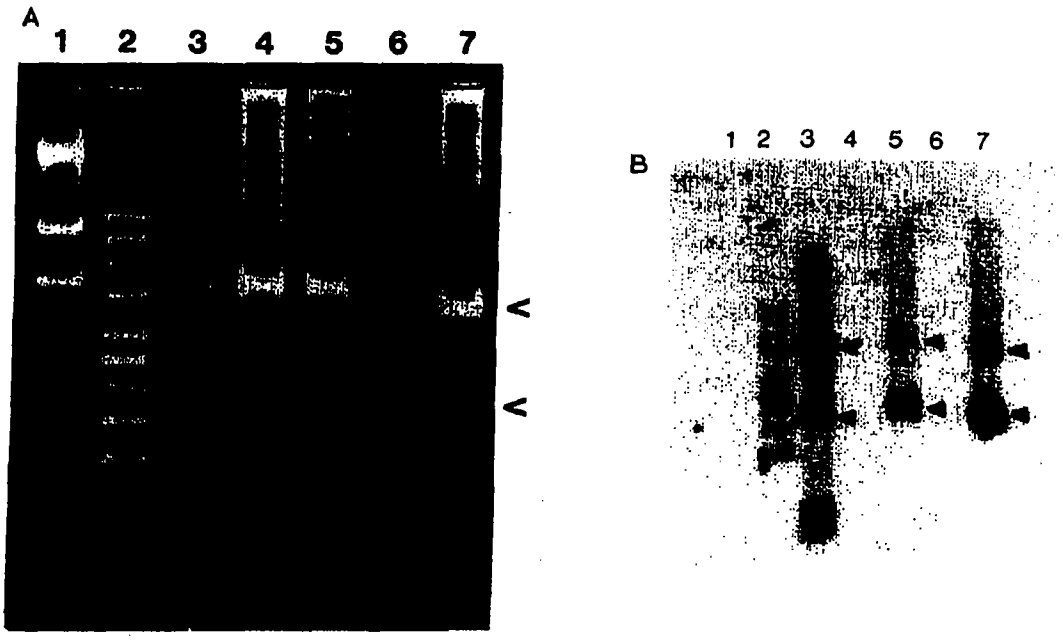

Fig. 2. The authenticity of pNZ17 transformants as shown by (A) ethidium bromide-stained agarose gel and (B) Southern hybridisation with ^{32}P-labelled pNZ17. Lanes: 1, λ/*Hin*dIII molecular mass markers; 2, BRL supercoiled plasmid DNA ladder; 3, *E. coli*/pNZ17; 4, *Lactobacillus fermentum* BR11; 5, *L. fermentum* BR11/pNZ17; 6, *L. acidophilus* BR9; 7, *L. acidophilus* BR9/pNz17. Arrows indicate pNZ17 bands.

3.4. Effect of post-pulse incubation time and selective media on electrotransformation efficiency

3.4.1. Post-pulse incubation time

Results of previous studies on the electroporation of plasmid DNA into industrial strains of lactobacilli have suggested that the post-pulse expression period required can vary depending upon the antibiotic resistance marker used [25]. We investigated the effect of post-pulse expression period (0–16 h) for the electrotransformation of pNZ17 (chloramphenicol resistance marker) and pSA3 (erythromycin resistance marker) into medically significant strains of lactobacilli. Our results show that whereas pNZ17 transformants expressing Cm^R were visible when plated immediately after the electroporation pulse, pSA3 transformants expressing Em^R required at least 2–3 h incubation in non-selective media before they were observed on recovery plates. The number of pSA3 transformants was found to be 60–80-times higher after 2–3 h post-pulse recovery time compared to no recovery time, but was not improved further by a 16 h post-pulse recovery period (data not shown).

3.4.2. Selective media

For plasmids pSA3 and pNZ17, the final number of transformants detected on each selective plate was influenced significantly by the concentration of the

Table 3
Effect of antibiotic concentration used in the selective plates on the recovery of electrotransformants using *Lactobacillus fermentum* BR11

Plasmid	Transformation efficiency (CFU/μg plasmid DNA)	
	Plated directly onto 3 μg/ml Cm	Plated on 0.5 μg/ml Cm prior to selection on 3 μg/ml Cm
pNZ17	2.6×10^3	4.5×10^4
pSA3	3.7×10^1	4.5×10^4

Following electroporation of *L. fermentum* with either pNZ17 or pSA3, we compared the effect of (a) plating directly onto selective levels of chloramphenicol (3 μg/ml) and (b) plating first onto the sub-inhibitory chloramphenicol concentration of 0.5 μg/ml and then replica plating onto 3 μg/ml to confirm high level expresion of the CAT gene. Results presented are from a single experiment.

antibiotic used. With chloramphenicol selection it was found that improved transformation efficiency was achieved if the electroporation mix first was plated onto agar with sub-inhibitory antibiotic concentrations (Table 3). This effect was even more noticeable with pSA3 when plated onto 0.5 μg/ml chloramphenicol and then replica plated onto 3.0 μg/ml chloramphenicol. This resulted in a 1000-fold increase in transformants obtained. The optimal concentration of antibiotic required in the selective media varied between lactobacillus strains. The optimal concentrations for *L. acidophilus* BR9 and *L. fermentum* BR11 were found to be 0.5 μg/ml of Cm and 1.5 μg/ml of Em, while for *Lactobacillus* sp. BR3 and *L. casei* ATCC 393 the optimal concentrations were 2.5 μg/ml of Cm and 2.5 μg/ml of Em. Replica plating onto media with higher antibiotic concentrations then was necessary to confirm the authenticity of transformants.

3.5. Summary: optimised protocol for the electrotransformation of lactobacilli with plasmid DNA

A 2% inoculum of an overnight *Lactobacillus* culture was grown in MRS broth for 2 h to reach an OD_{660} of 0.1–0.2. Penicillin then was added to a final concentration of 10 μg/ml and the incubation continued for a further 60–90 min. The cells were harvested, washed twice in ice-cold washing buffer (5 mM sodium phosphate, 1 mM $MgCl_2$) and resuspended to 1% of the original culture volume in ice-cold electroporation buffer (0.9 M sucrose, 3 mM $MgCl_2$, pH 7.4). Plasmid DNA (0.5 μg in 1 μl) was mixed with 50 μl of ice-cold cell suspension in a 0.2 cm Gene Pulser™ cuvette and held on ice for at least 2 min. This mixture then was exposed to a high voltage electric pulse (peak voltage of 2.5 kV, capacitance of 25 μF, parallel resistance of 400 Ω) which delivered a peak field strength of 12.5 kV/cm. Following the pulse, the cell suspension was diluted 10-fold with MRS broth and incubated (in the absence of antibiotic selection) at 37°C for 2–3 h. The diluted suspension then was plated onto MRS agar plates containing the sub-inhibitory concentration of chloramphenicol of 0.5 μg/ml (for both pNZ17 and pSA3). After 48 h incubation, potential pNZ17 and pSA3 transformants were replica plated onto plates containing 3.0 μg/ml chloramphenicol for confirmation.

4. Discussion

Our early attempts at electroporation with four *Lactobacillus* strains of medical significance resulted in transformation efficiencies which varied significantly on a daily basis, from zero up to 180 transformants/μg DNA. Because this low and variable transformation rate is unsatisfactory for cloning and other genetic manipulations, we focused our attention on factors influencing electrotransformation of lactobacilli in an attempt to decrease the variability and to increase the overall transformation efficiency for this genus. Of the many factors which affect the electroporation efficiency of gram-positive bacteria, the structure and composition of the cell wall is critical [31–33]. The problem of permeating the rigid structure of the Gram-positive cell wall can be overcome in two ways. Firstly, higher field strength during electroporation can be useful for introducing holes into the cell wall so that recipient cells can reach the critical transmembrane potential to become permeabilized. We found that a peak field strength of 12.5 kV/cm was successful with our lactobacilli. The second approach is to chemically or enzymatically weaken the cell wall prior to electroporation. Cell wall damage, caused either by catabolic enzymes or by incorporating penicillin or glycine in the growth media, has been shown previously to improve transformation efficiencies in several Gram-positive bacteria [25,31,34,35]. Our studies have shown that all three methods can be used to improve the electrotransformation efficiencies of medically-important lactobacilli. We achieved a 10–150-fold increase in transformation efficiency either by treating the recipient *Lactobacillus* cells with lysozyme (10 μg/ml) or by incorporating 1% glycine into the culture media. Even better transformation efficiencies were obtained if the cells were pre-treated with penicillin. In addition to increasing the transformation efficiency, the more important finding was that increased transformation efficiencies of even normally refractory strains consistently could be achieved, provided that the cell wall was weakened in some manner.

Another feature of the electroporation conditions used in this study was the requirement for an expression period following exposure to the electric pulse with pSA3 but not pNZ17. This difference can be explained by the fact that pNZ17 transformants are selected on chloramphenicol plates and that the CAT gene is rapidly expressed following resumption of bacterial protein synthesis. In comparison, pSA3 transformants are selected on erythromycin plates and resistance to this antibiotic is known to involve a post-transcriptional regulatory mechanism [36]. This mechanism involves the conformational modification of a methylase mRNA which subsequently is responsible for the resistance phenotype. It is not surprising therefore that this series of events requires several hours of expression before fully-resistant transformants are present and able to grow on erythromycin plates. This also explains why extending the post-pulse recovery period up to 16 h does not further increase the number of transformants obtained. Our results with *Lactobacillus* strains of medical significance support those of Posno et al. [25] who focused primarily on *Lactobacillus* strains of industrial significance.

As has been reported by others, the strain of recipient bacterium significantly

affects the electrotransformation efficiency. All three guinea pig vaginal isolates were much more difficult to electrotransform than the human *L. casei* isolate. However, by adopting our optimised protocol we consistently were able to obtain electrotransformation efficiencies of up to 10^4 CFU/μg DNA with all *Lactobacillus* strains tested. The availability of a reproducible and efficient transformation system for lactobacilli should facilitate the cloning of foreign genes into medically and industrially important bacteria and enable subsequent evaluation of gastrointestinal and vaginal lactobacilli as potential live vaccine vehicles.

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Molecular Cloning and Deoxyribonucleic Acid Polymorphisms in *Lactobacillus acidophilus* and *Lactobacillus gasseri*[1]

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ABSTRACT

Lactobacillus strain ADH is a bile-resistant, bacteriocin-producing human isolate that was phenotypically classified within the *Lactobacillus acidophilus* group. Total DNA and phage DNA extracted from strain ADH were separately digested with *BclI* and ligated with *BclI*-digested pGK12. Following electroporation of these ligation mixtures directly into strain ADH, electrotransformants were recovered at frequencies of 1.5×10^3 and $2.0 \times 10^4/\mu g$ of pGK12 for preparations of pGK12::phage DNA and pGK12::total DNA, respectively. Among the electrotransformants screened, 6 and 22% contained passenger DNA of either phage DNA or chromosomal origin, respectively, as determined by restriction-enzyme analyses and hybridization assays. Derivatives of pGK12 containing passenger DNA of chromosomal (pTRK120) or phage (pTRK121) origin and pTRK15 (native cryptic plasmid) were evaluated for use as species-specific probes. The strain ADH-derived probes hybridized primarily to members of the B-1 and B-2 lactobacilli homology groups and demonstrated strain-specific polymorphisms within these groups. Identical hybridization patterns were established for strain ADH and *Lactobacillus gasseri* VPI 6033 (ATCC 19992). Identification of DNA probes and establishment of a host-vector cloning system have facilitated our efforts to characterize the *Lactobacillus* chromosome and to distinguish between closely related species thought to be important inhabitants of the gastrointestinal tract.
(Key words: *Lactobacillus*, probes, polymorphisms)

Abbreviation key: Cm^r = resistant to chloramphenicol, Em^r = resistant to erythromycin, Em^s = sensitive to erythromycin, **RFLP** = restriction fragment length polymorphism.

INTRODUCTION

Lactobacillus acidophilus colonizes the keratinized squamous epithelium of man and animals in a host-specific manner (38, 43). In dairy product applications, *L. acidophilus* is made available to the consumer as a dietary adjunct in acidophilus milk (40); however, the therapeutic and gastrointestinal roles of dietary lactobacilli and fermented products derived thereof have been the subject of much debate (6, 8, 14, 38, 42). Because of the practical applications to the dairy industry, as well as the implied clinical significance, we have directed our efforts to characterize genetically the strains that are phenotypically classified as *L. acidophilus*. Research emphasis has been directed previously toward characterization of plasmid-associated phenotypes of lactobacilli; thus, the *Lactobacillus* chromosome remains largely unexplored. In an effort to exploit the chromosomal complement of genetic information more thoroughly, we have initiated efforts to isolate and characterize sequences of chromosomal origin.

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EXHIBIT C

Lactobacillus acidophilus constitutes a genotypically heterogenous group of organisms, many of which are often difficult to differentiate solely on phenotypic characteristics (13, 18, 19). For example, *Lactobacillus gasseri* strains have been cataloged as *L. acidophilus* strains because these two species are phenotypically indistinguishable by classical methods. Similarly, it remains unclear which species (if not both) maintains the dominant role in the intestinal tract (18).

Methods that have been used to identify and classify lactobacilli include susceptibility to antimicrobial agents, plasmid fingerprinting, serological examination, DNA hybridization analyses, and various physiological, biochemical, and fermentation tests (2, 12, 13, 36, 39). Serious constraints are placed on the reliability of these methods by mutations, antigenic variations, and dissemination or loss of plasmids. Although efforts have been made to classify lactobacilli by restriction endonuclease patterns (41) and DNA probe technologies (4, 30, 31), restriction fragment length polymorphism (RFLP) analysis has not been exhaustively evaluated as an alternative method to identify, characterize, and differentiate *Lactobacillus* spp. This technology has been used, however, to study the epidemiology and molecular biology of other genera (1, 5, 10, 25, 27, 29, 35, 37). Briefly, RFLP analysis defines alterations in a limited number of homologous restriction fragments using specific probes to detect obscure differences among otherwise closely related strains. A practicable RFLP typing system and species- or strain-specific DNA probes would greatly facilitate genetic analysis and provide a wealth of fundamental information regarding the taxonomy and chromosomal organization of the *L. acidophilus* group.

We have worked extensively with *Lactobacillus* strain ADH. Strain ADH was classified previously as *L. acidophilus* by phenotypic and physiological characteristics. This strain was initially selected by screening putative *L. acidophilus* culture repositories for strains with characteristics presumably important to intestinal maintenance and activity [e.g., in vitro adherence to human fetal intestinal cells; (16)]. Subsequent investigations revealed that strain ADH tolerated pH, survived stomach passage, and adhered to intestinal cells better than other strains, including the *L. acidophilus* strain currently used in "acidophilus" milk products (3).

In the present study, pGK12-based recombinant plasmids were directly recovered in *Lactobacillus* strain ADH by electroporation. In addition to sequences from the native temperate phage (ϕadh), fragments of chromosomal DNA from strain ADH were also cloned into plasmid pGK12. We report herein on the molecular cloning and evaluation of selected recombinant plasmids for the identification, classification, and genomic analysis of *Lactobacillus* spp. These analyses revealed that *Lactobacillus* strain ADH and *L. gasseri* VPI 6033 (ATCC 19992) are isogenic.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids

The bacteria and plasmids used in this study are listed in Table 1. Bacterial strains were maintained as described previously (22). The inducible prophage (ϕadh) from *Lactobacillus* strain ADH was recently characterized morphologically, physically, and genetically (33). Plasmid pGK12 is a well-characterized, small (4.4-kb) vector containing two antibiotic resistance determinants [chloramphenicol resistance (Cm^r; *cat* gene) from pC194; erythromycin resistance (Em^r; *ermC* gene) from pE194] and the pWV01 (small cryptic plasmid native to *Lactococcus lactis* spp. *cremoris*) origin of replication, which is functional in a wide variety of Gram-positive bacteria (23) as well as in *Escherichia coli* (17).

Restriction Enzyme Analysis

Restriction enzymes purchased from BRL (Bethesda Research Laboratories, Gaithersburg, MD), IBI (International Biotechnologies, Inc., New Haven, CT), and Boehringer Mannheim (Indianapolis, IN) were used as recommended by the suppliers to digest all DNA to completion.

DNA Isolation and Purification

Plasmid DNA was isolated and purified through CsCl-ethidium bromide density gradients as described previously (22). Phage ϕadh DNA was isolated from lysates and purified as described (33).

TABLE 1. Bacterial strains and plasmids.

Bacterial strains		Relevant characteristics[1]	Origin or reference
Lactobacillus strain ADH			
NCK101		ϕadh^+, *str-10 spc-11* (pTRK15)	(22)
NCK102		ϕadh^-, (pTRK15)	(33)
NCK110		ϕadh^+, *gnt-12 rif-13* (pTRK15)	Klaenhammer[2]
NCK111		ϕadh^+, *str-10 spc-11* (pTRK15) (pGK12)	(22)
NCK220		ϕadh^+, *str-10 spc-11* (pTRK15) (pTRK120)	This study; NCK101 derivative
NCK221		ϕadh^+, *str-10 spc-11* (pTRK15) (pTRK121)	This study; NCK101 derivative
Lactobacillus acidophilus			
NCFM/N2		Smooth colony isolate of RL8K	(15)
ATCC 4356		Neotype *L. acidophilus*, DSM 20079	(18)
VPI No.[3]	Homology groups		
6032	A-1	ATCC 4356	(12)
11084	A-1	NCTC 1899	(12)
7635	A-2		(12)
11083	A-2	NCTC 2949	(12)
1754	A-3		(12)
1756	A-3		(12)
1830	A-3		(12)
1793	A-4		(12)
6033	B-1[4]	ATCC 19992	(12)
11089	B-1[4]	ATCC 9857	(12)
11092	B-1[4]	ATCC 29601	(12)
12601	B-1[4]	YIT-0164	(12)
11088	B-2	ATCC 11506, NCK88[2]	(12)
11694	B-2	NCTC 1406	(12)
11696	B-2	NCTC 1407	(12)
Lactobacillus gasseri[4]			
ATCC 33323		63AM Gasser, DSM 20243	(19)
Enterococcus faecalis 19433			Klaenhammer[2]
Escherichia coli DH5α			Stratagene (La Jolla, CA)
Lactobacillus helveticus 481			(11)
Lactococcus lactis MG1363		Plasmid free	(7)
Staphylococcus aureus NCTC 8325		JBL71	(20)
Plasmids			
pGK12		Cm^r, Em^r, 4.4 kb	(17)
pTRK15		cryptic plasmid in ADH, 26.5 kb	(22)
pTRK120		Cm^r, Em^s, 5.7 kb	This study[5]
pTRK121		Cm^r, Em^s, 5.9 kb	This study[6]

[1]ϕadh^+, ϕadh lysogen; ϕadh^-, cured of the ϕadh prophage; *str-10*, streptomycin resistance (1 mg/ml); *spc-11*, spectinomycin resistance (300 μg/ml); *gnt-12*, gentamicin resistance (200 μg/ml); *rif-13*, rifamycin resistance (25 μg/ml); Cm^r, resistant to chloramphenicol; Em^r, resistant to erythromycin; Em^s, sensitive to erythromycin.

[2]NCK Culture Collection of T. R. Klaenhammer, Department of Food Science, North Carolina State University, Raleigh, NC.

[3]VPI = Virginia Polytechnic Institute. Strain numbers for cultures maintained in the collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, VA.

[4]The B-1 group strains of Johnson et al. (12) are >50% homologous to the IIa group that Lauer et al. (18) defined as *L. gasseri*.

[5]A recombinant plasmid composed of a 1.3-kb *BclI* fragment of random chromosomal DNA from *L. acidophilus* ADH ligated to the 4.4-kb pGK12 *BclI* fragment.

[6]A recombinant plasmid composed of a 1.5-kb *BclI* fragment of ϕadh DNA ligated to the 4.4-kb pGK12 *BclI* fragment.

Total DNA was extracted from *Lactobacillus* spp. and *Enterococcus faecalis* as follows. Cells from an overnight culture previously grown at 37°C in 100 ml of MRS broth (Difco Laboratories, Detroit, MI) were collected by centrifugation and resuspended in 200 ml of fresh MRS broth. Following a 2-h incubation at 37°C, the cells were harvested by centrifugation, washed twice in TES buffer (50 m*M* NaCl, 30 m*M* Tris, pH 8.0, and 5 m*M* EDTA), and resuspended in 1 ml of lysis buffer containing mutanolysin (40 μg/ml; Sigma Chemical Co., St. Louis, MO) and lysozyme (20 mg/ml; Sigma). The lysis buffer consisted of 25% sucrose (ultrapure; BRL), 50 m*M* Tris, and 1 m*M* EDTA, pH 8.0. This mixture was incubated at 37°C for 45 min, and then 1 ml of .25 *M* EDTA (pH 8.0) was added, followed by an additional 5-min incubation at room temperature. A 400-μl volume of 20% SDS was added, and the mixture was allowed to incubate at 65°C until the solution cleared. After the addition of 20 μl of proteinase K (20 mg/ml; Sigma) and another 15-min incubation at 65°C, the lysate was extracted twice with an equal volume of phenol and once with an equal volume of chloroform-isoamyl alcohol (24:1). Following the addition of 2 volumes of cold (–20°C) 95% ethanol, the DNA was spooled onto a glass rod, submersed in cold (–20°C) 70% ethanol, dissolved in a nominal volume of TE (10 m*M* Tris hydrochloride, pH 8.0, and 1 m*M* EDTA), and stored at 4°C until used.

Total DNA was extracted from *Staphylococcus aureus* and *Lactococcus lactis* spp. *lactis* as described by Luchansky et al. (20) and Hill et al. (9), respectively.

Electrotransformation, Molecular Cloning, and DNA Hybridizations

Electroporation experiments were conducted essentially as described by Luchansky et al. (21) with a Gene Pulser™ apparatus (Bio-Rad Laboratories, Richmond, CA) using .4-cm interelectrode gap cuvettes, large volume washes [300 ml of 3.5 times SMEB (1×, 272 m*M* sucrose, 1 m*M* $MgCl_2$, pH 7.2) per cell pellet recovered from 100 ml of original cells], a single pulse of 6250 V/cm at 25 uFD capacitance, and a series resistor (5-ohm box). Ligations of DNA molecules were performed as suggested by the supplier of T_4 DNA ligase (BRL). Hybridization reactions and autoradiography were performed as described previously (20). When used as probes, pTRK120 and pTRK121 were extracted from *E. coli* DH5α to preclude spurious background hybridization.

RESULTS

Cloning φadh and Random Chromosomal Sequences

Passenger DNA was cloned into the unique *BclI* site in the *ermC* gene of pGK12. Total DNA extracted from *Lactobacillus* strain ADH (Chr) and phage φadh DNA were each digested with *BclI* and separately ligated to *BclI*-digested pGK12. These ligation mixtures were electrotransformed into strain ADH; selection was made for resistance to 7.5 μg/ml of chloramphenicol. As shown in Table 2, Cm^r electrotransformants of the control DNA (uncut pGK12) were recovered at a frequency of 5.6×10^5/μg. The pGK12::φadh and pGK12::Chr ligation mixtures yielded Cm^r electrotransformants at frequencies per microgram of vector pGK12 of 1.5×10^3 and 2.0×10^4, respectively. Of the Cm^r electrotransformants screened from the pGK12::φadh and pGK12::Chr clones recovered, 6% (14/240) and 22% (10/46), respectively, were sensitive to erythromycin (Em^s). Restriction endonuclease and hybridization analyses demonstrated that the passenger DNA harbored by Cm^r Em^s elec-

TABLE 2. Cloning in *Lactobacillus* strain ADH via electroporation.

Ligation mixtures[1]	Transformants per microgram of DNA	Cm^r Em^r	Cm^r Em^s
		(%)	
pGK12 (uncut)	5.6×10^5	100	0
pGK12::Chr	2.0×10^4	78[2]	22[2]
pGK12::φadh	1.5×10^3	94[3]	6[3]

[1]Ligation mixtures consisted of *BclI*-digested pGK12 ligated to either *BclI*-digested phage φadh fragments (pCK12::φadh) or *BclI*-digested chromosomal fragments from strain ADH (pGK12::Chr).

[2]46 colonies screened.

[3]240 colonies screened.

supplier of T_4 DNA ligase
tion reactions and autoradi-
formed as described previ-
used as probes, pTRK120
ere extracted from *E. coli*
e spurious background hy-

RESULTS

Random ences

. was cloned into the unique
mC gene of pGK12. Total
m *Lactobacillus* strain ADH
ϕadh DNA were each
I and separately ligated to
12. These ligation mixtures
ormed into strain ADH;
for resistance to 7.5 μg/ml
l. As shown in Table 2, Cm^r
s of the control DNA (un-
recovered at a frequency of
pGK12::ϕadh and pGK12::
es yielded Cm^r electrotrans-
icies per microgram of vec-
10^3 and 2.0×10^4, respec-
Cm^r electrotransformants
pGK12::ϕadh and pGK12::
ed, 6% (14/240) and 22%
y, were sensitive to erythro-
triction endonuclease and
ses demonstrated that the
rbored by Cm^r Em^s elec-

actobacillus strain ADH via elec-

ormants icrogram lA	Cm^r Em^r	Cm^r Em^s
	(%)	
10^5	100	0
10^4	78[2]	22[2]
10^3	94[3]	6[3]

onsisted of *Bcl*I-digested pGK12
ligested phage ϕadh fragments
ligested chromosomal fragments
12::Chr).
d.
ed.

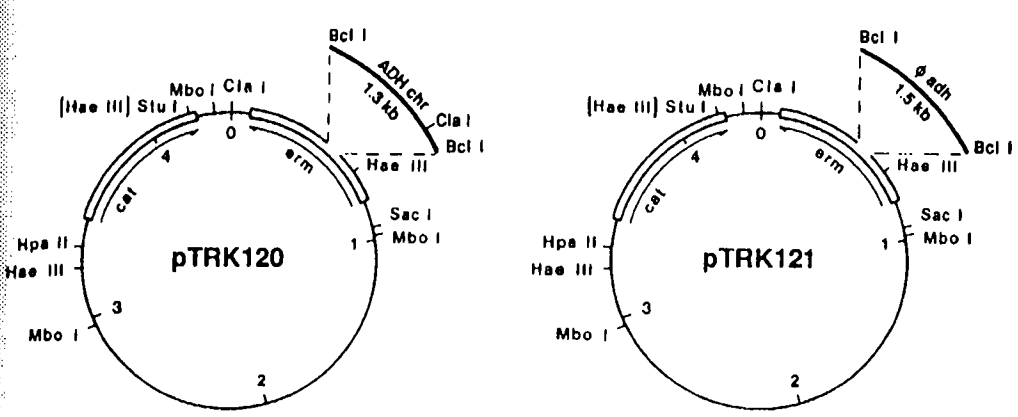

Figure 1. Genetic and physical maps of pTRK120 and pTRK121. Plasmid pTRK120 is pGK12 (thin line) containing a 1.3-kb *Bcl*I fragment (ADH chr) from the chromosome of *Lactobacillus* strain ADH (thick line). Plasmid pTRK121 is pGK12 (thin line) containing a 1.5-kb *Bcl*I fragment (ϕadh) of bacteriophage ϕadh (thick line). The size of the cloned *Bcl*I fragments in pTRK120 and pTRK121 are not drawn to scale. Figures modified from Kok et al. (17).

trotransformants were of chromosomal (pGK12::Chr) or ϕadh (pGK12::ϕadh) origin (data not shown). Figure 1 shows representative recombinant plasmids. Plasmid pTRK120 contains a 1.3-kb *Bcl*I fragment of chromosomal origin, and pTRK121 contains a 1.5-kb *Bcl*I fragment from ϕadh.

Evaluation of pTRK120 and pTRK121 for Use as Genetic Probes for *Lactobacilli*

Recombinant plasmids pTRK120 and pTRK121 were evaluated as genetic probes for specificity to lactobacilli strains. As shown in Table 3, both probes hybridized to strain ADH and *L. gasseri* VPI 6033 (ATCC 19992), but neither pTRK120 nor pTRK121 hybridized to total DNA from heterologous hosts, most notably *L. acidophilus* ATCC 4356. These data suggested that pTRK120 and pTRK121 were more homologous with *L. gasseri* than neotype *L. acidophilus*.

To confirm our preliminary results, total DNA was extracted from a variety of *L. acidophilus* and *L. gasseri* strains and separately hybridized to ^{32}P-labeled pTRK120, pTRK121, and the native cryptic plasmid of

TABLE 3. Evaluation of pTRK120 and pTRK121 as genetic probes for *Lactobacillus*.

Source of DNA[1]	Hybridization with[2] pTRK120	pTRK121
Lactobacillus acidophilus ATCC 4356	–	–
Lactobacillus gasseri ATCC 19992[3]	+++	+++
Lactobacillus strain ADH	+++	+++
Enterococcus faecalis 19433	–	–
Lactobacillus helveticus 481	–	–
Lactococcus lactis MG1363	–	–
Staphylococcus aureus NCTC 8325	–	–

[1]Total DNA was extracted from each culture and digested with *Eco*RI. The resulting fragments were fractionated by agarose gel electrophoresis, electrotransferred to Magnagraph membranes (Micron Separations, Inc., Westboro, MA), and hybridized to labeled pTRK120 or pTRK121.

[2]Hybridization to probes denoted by +++; absence of hybridization to probes denoted by –.

[3]Listed as *L. acidophilus* VPI 6033 (ATCC 19992; B-1 homology group) by Johnson et al. (12).

TABLE 4. Evaluation of pTRK15, pTRK120, and pTRK121 as genetic probes.

Homology group	Designation[1]	Hybridization with[2] pTRK15	pTRK120	pTRK121
ND[3]	ADH (NCK102)	+++	+++	–
ND	ADH (NCK110)	+++	+++	+++
ND	NCFM/N2[4]	–	–	–
A-1	6032	–	–	–
	11084	–	–	–
A-2	11083	+++	–	+
	7635	–	–	–
A-3	1754	–	–	–
	1756	–	–	–
	1830	–	+++	–
A-4	1793	–	–	–
B-1[5]	63AM (ATCC 33323)	ND	+	–
	6033 (ATCC 19992)	+++	+++	+++
	11089 (ATCC 9857)	ND	+++	+++
	11092	–	+++	+++
	12601	–	+++	+++
B-2[6]	11088 (ATCC 11506)	ND	++	–
	11694	–	–	+++
	11696	–	–	+++

[1]Virginia Polytechnic Institute numbers for strains within the homology groups defined by Johnson et al. (12). Total DNA extracted from each strain was digested with *Eco*RI. The resulting fragments were fractionated by agarose gel electrophoresis for hybridization analyses.

[2]Hybridization to probes denoted by +++; absence of hybridization to probes denoted by –; weak and medium hybridization denoted by + and ++, respectively.

[3]ND = Not determined.

[4]N2 is a single colony isolate from *Lactobacillus acidophilus* RL8K (15).

[5]Homology group IIa of Lauer et al. (18).

[6]Homology group IIb of Lauer et al. (18).

strain ADH, pTRK15. The pTRK120 probe hybridized strongly to the control DNA (NCK102 and NCK110) and to VPI strains 6033, 11089, 11092, and 12601 of the B-1 homology group (Table 4). Strong signals were also detected for two strains outside the B-1 group, VPI 1830 (A-3 group) and VPI 11088 (B-2 group). A weaker, but definitive signal was also detected in neotype *L. gasseri*, strain ATCC 33323. Plasmid pTRK15 hybridized strongly to total DNAs from strain ADH (NCK102 and NCK110) and to strains VPI 6033 (B-1 homology group) and VPI 11083 (A-2 homology group). Plasmid pTRK121 (containing ϕadh DNA) shared homology with all B-1 group strains tested except neotype *L. gasseri* (ATCC 33323). Plasmid pTRK121 also hybridized strongly with total DNA from strains VPI 11694 and VPI 11696 (B-2 homology group) and showed weak homology with VPI 11083 (A-2). As expected, pTRK121 hybridized with DNA sequences from the ϕadh lysogen (NCK110) but not to sequences from the prophage-cured derivative of strain ADH (NCK102).

Identification of DNA Polymorphisms Among Strains in B-1 and B-2 Homology Groups

To define further the genotypic relatedness among strain ADH and other lactobacilli, plasmids pTRK120 and pTRK121 were used as probes for hybridization to restriction endonuclease-digested total DNA from selected representatives of the B-1 and B-2 homology groups of Johnson et al. (12). Plasmid pTRK121 shared homology with total DNA extracted from all strains tested except for NCK102, the prophage-cured derivative of strain ADH (Figure 2A). Hybridization of ^{32}P-labeled pTRK121 to *Eco*RI-digested chro-

bridization with[2]	
pTRK120	pTRK121
+++	–
+++	+++
–	–
–	–
–	–
–	+
–	–
–	–
–	–
+++	–
–	–
+	–
+++	+++
+++	+++
+++	+++
+++	+++
++	–
–	+++
–	+++

:fined by Johnson et al. (12). Total
: were fractionated by agarose gel

denoted by –; weak and medium

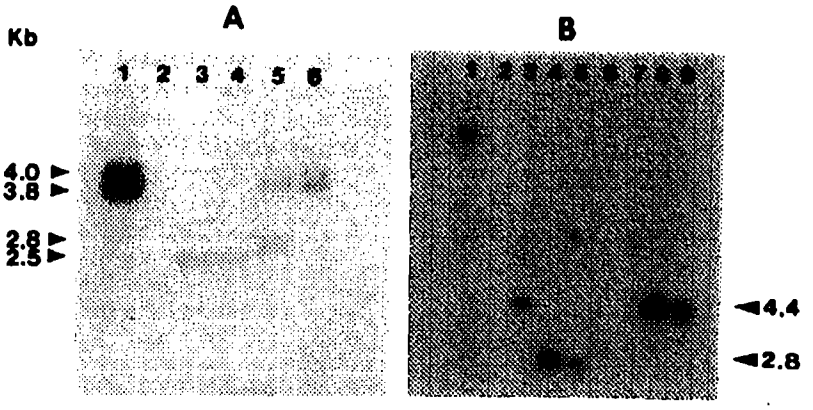

Figure 2. Hybridization of ^{32}P-labeled pTRK121 (panel A) and ^{32}P-labeled pTRK120 (panel B) to *Eco*RI-digested total DNA extracted from derivatives of strain ADH and *Lactobacillus* strains from various homology groups (in parentheses). Panel A: probe pTRK121; contains phage ϕadh DNA. Lane 1, NCK110 (ϕadh lysogen); lane 2, NCK102 (prophage-cured derivative of strain ADH); lane 3, VPI 11696 (B-2); lane 4, VPI 11694 (B-2); lane 5, VPI 11092 (B-1); lane 6, VPI 6033 (B-1). Panel B: probe pTRK120; contains strain ADH chromosomal DNA. Lane 1, VPI 1830 (A-3); lane 2, VPI 1793 (A-4); lane 3, VPI 6033 (B-1); lane 4, VPI 11092 (B-1); lane 5, VPI 12601 (B-1); lane 6, VPI 11694 (B-2); lane 7, VPI 11696 (B-2); lane 8, NCK102 (prophage cured); lane 9 NCK110 (ϕadh lysogen).

NA sequences from the ϕadh
but not to sequences from
l derivative of strain ADH

A Polymorphisms
-1 and B-2

er the genotypic relatedness
and other lactobacilli, plas-
id pTRK121 were used as
zation to restriction endonu-
l DNA from selected repre-
B-1 and B-2 homology
on et al. (12). Plasmid
homology with total DNA
l strains tested except for
phage-cured derivative of
ure 2A). Hybridization of
21 to *Eco*RI-digested chro-

mosomal DNA from strains VPI 11694 and VPI 11696 identified a common fragment of similar size in each strain, approximately 2.5 kb. The pTRK121 probe hybridized with two bands (approximately 2.8 kb and 3.8 kb) in VPI 11092 (B-1 homology group). Two bands, approximately 3.8 kb and 4.0 kb, were also visible following hybridization of pTRK121 to *Eco*RI-digested total DNA from the other B-1 group strain tested, VPI 6033 (*L. gasseri* ATCC 19992). More importantly, the *Eco*RI chromosomal sequences hybridizing with pTRK121 in VPI 6033 occupied the same relative position (migrated the same distance) as the *Eco*RI fragments of strain ADH (NCK110) that hybridized with pTRK121.

Similar results were obtained using plasmid pTRK120 as a probe (Figure 2B). Single *Eco*RI fragments about 4.4 kb in length were detected in both ADH derivatives (NCK102 and NCK110; lanes 8 and 9) and VPI 6033 (*L. gasseri* ATCC 19992). A smaller *Eco*RI fragment (about 2.8 kb) hybridized with pTRK120 in two other B-1 group strains, VPI 11092 and VPI 12601 (lanes 4 and 5). However, the strain ADH-derived probe prepared from chromosomal DNA (pTRK120) was not specific for strains of the B-1 homology group, because this probe also shared homology with a large *Eco*RI fragment in VPI 1830, an A-3 group strain (Figure 2B, lane 1).

Hybridization of ^{32}P-labeled pTRK15 with *Eco*RI-digested total DNA from strain ADH (NCK102 and NCK110) and strain VPI 6033 (*L. gasseri* ATCC 19992) revealed identical hybridization patterns among all three strains (data not shown).

DISCUSSION

Despite the availability of gene transfer systems for *Lactobacillus* species (21, 24, 26, 28, 33), very little information has accumulated concerning the genomic organization of these bacteria. The chromosome is a largely unexplored repository of genetic information that must be defined in order to realize fully the improvement or diversification of strains through genetic technologies. In this investigation, we report the application of electroporation for introducing recombinant plasmids into *Lactobacillus* strain ADH and the genetic relatedness of this strain with *L. gasseri* VPI 6033 (ATCC 19992). Three distinct probes (pTRK15, pTRK120, and pTRK121) hybridized in identical fashion to strains ADH and VPI 6033. The specificity of these probes and accompanying polymorphisms establishes une-

quivocal genetic relationships among otherwise indistinguishable strains or species with untraceable lineages due to coding ambiguities. Heretofore, we recommend that *L. acidophilus* ADH be designated *L. gasseri* ADH.

Experiments that examined the efficiency of shotgun cloning chromosomal sequences into *L. gasseri* ADH via electroporation were successful in that recombinant plasmids were recovered. Ligation mixtures generated electrotransformants of strain ADH at relatively high frequency (10^3 to 10^4/μg of pGK12) and recombinant moieties at high efficiency (6 to 22%). However, a 1- to 2-log decrease in the frequency of electroporation was observed using pGK12-based ligation mixtures compared with uncut preparations of pGK12 (Table 2). Similar results were obtained with *Lactococcus lactis* spp. *lactis*; about a 1-log decrease in frequency was observed when using ligation mixtures for electroporation rather than covalently closed circular DNA (32). These data indicate the importance of optimizing conditions for electroporation for maximal recovery of electrotransformants to ensure recovery of recombinant-containing transformants from ligation mixtures.

The recovery of plasmids pTRK120 and pTRK121 represents one of the first reports of direct cloning in *Lactobacillus* via electroporation. Our results compare favorably with those for *Lactococcus lactis* spp. *lactis* in which plasmid molecules were recovered at a frequency of 5.3×10^3/μg of pGK12 using ligation mixtures for electroportion (32). Electrotransformation is rapidly becoming the tool of choice for gene transfer experiments, being favored over more conventional methodologies such as competent cell and protoplast transformation because it is often more efficient, rapid, facile, and less tedious. The use of *L. gasseri* ADH as a cloning host obviates the requirement for primary cloning into either *E. coli* or more well-characterized Gram-positive organisms (e.g., *Bacillus subtilis* or *Lactococcus lactis* spp. *lactis*).

The six *L. acidophilus* homology groups (A-1 to A-4, B-1, and B-2) described by Johnson et al. (12) comprise a heterogeneous collection of "acidophilus-type" strains and species that are difficult to separate solely by phenotype (13, 18, 19). Several investigators (4, 30, 31, 41) have developed nucleic acid probes for identification of lactobacilli, including *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus reuteri*. As one aim of this study, we evaluated DNA probes with specificity for strains or species in the *L. acidophilus* and *L. gasseri* groupings. The chromosomal (pTRK120) and phage (pTRK121) sequences from strain ADH reacted primarily with *L. gasseri* strains in the B-1 homology group. These same probes hybridized to varying degrees with strains in the A-2 (pTRK121), A-3 (pTRK120), and B-2 (pTRK120 and pTRK121) groups, thus indicating that these probes are not specific for select *L. gasseri* strains (homology group B-1). The heterogeneity of the *L. acidophilus* and *L. gasseri* species, however, may again be reflected in the range of reactions that we detected with the chromosomal and phage probes used in this study.

The *L. acidophilus* B-1 and B-2 homology groups first delineated by Johnson et al. (12) and classified concurrently as groups IIa and IIb, respectively, by Lauer et al. (18) are phenotypically indistinguishable; these groups are presently classified as a separate species, *L. gasseri*. The "I" homology groups of Lauer et al. (18) retain the name *L. acidophilus* because this group contains the neotype strain (ATCC 4356). However, within group "I" *L. acidophilus* strains, there are five subgroups that are only 30 to 50% homologous at the DNA level. The intestinal roles and relative significance among these strains remain undefined. Similarly, the in vivo significance of *L. gasseri* strains relative to *L. acidophilus* subgroups has not been established.

The B-2 (IIb) group of *L. gasseri* are only 30 to 50% homologous with the B-1 (IIa) group. Lauer et al. (18) indicated group IIb will occupy a separate taxon when a distinguishing phenotype is defined. In this regard, it is interesting that pTRK120 and pTRK121 reacted with strains in both the B-1 and B-2 homology groups. Recently, phage ϕadh has been shown to exhibit transduction to selected strains within both the B-1 and B-2 groups of *L. gasseri* (34). This suggests that ϕadh, remnants thereof, or other prophages related to ϕadh are distributed among homology groups comprising *L. gasseri* strains. Their common phenotypes and gastrointestinal habitat further

ion of lactobacilli, includ-
rvatus, Lactobacillus del-
lus helveticus, and *Lac-*
; one aim of this study, we
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indicate that the two subgroups of *L. gasseri* may be functionally related and exchange genetic information in a common ecological niche.

The Lactobacillaceae have found extensive use in dairy products and dairy processing. Additionally, association of lactobacilli with the intestinal tract of mammals has been implicated as beneficial to maintenance of a healthful microbial balance (6, 8, 42). Past emphasis for strain improvement has largely involved screening natural isolates for desired characteristics and monitoring existing strains for beneficial adaptations. Molecular approaches provide a powerful complement to more traditional methods for studying the genus *Lactobacillus* and for defining the roles and relative significance of those subgroups now named as *L. acidophilus* and *L. gasseri*. Utilization of electroporation to recover recombinant molecules in lactobacilli will provide new opportunities for directed modifications of these organisms. The use of pTRK120, pTRK121, or similar plasmids as genetic probes will facilitate strain isolation, identification, and classification. Analyses of chromosomal sequences by direct cloning will provide a wealth of basic information concerning the genetics and molecular biology of these bacteria. The accumulation of fundamental knowledge obtained with a few model strains will in turn have a profound impact on unraveling the gastrointestinal functionality of lactobacilli and on genetic engineering of cultures having commercial potential or significance.

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